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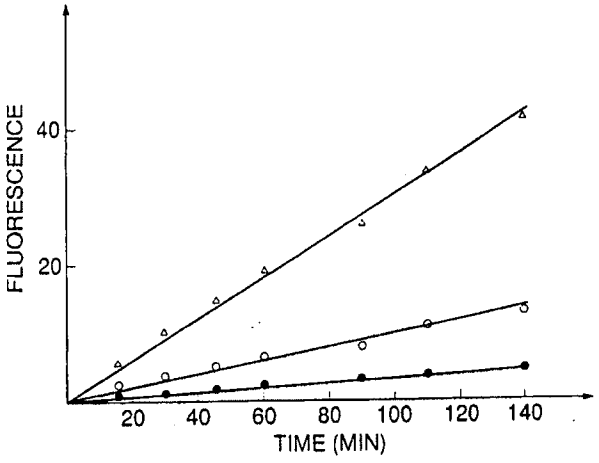
(54) Direct method for detecting very low levels of coliform contamination.

(57) A direct method for detecting very low levels of coliform contamination in products for human consumption comprises contacting the microorganisms with a methylumbelliferone substrate. The substrate is hydrolyzed into methylumbelliferone by an enzyme given off by the microorganisms. Hydrolysis is accelerated by sodium lauryl sulfate, which renders the microorganisms more permeable to the substrate, the enzyme, or both. The methylumbelliferone is detected by its fluorescence, either in solution or on an agar medium supporting microcolonies formed from individual microorganisms.

FIG. 1.



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Related References: This application is a divisional application of EPO-application No. 88909391.0.

This invention relates to processes for detecting coliform bacteria, and more specifically has reference to processes for such detection in products for human consumption which operate in a sufficiently rapid time as to allow treatment of the product before such consumption, if needed.

It has long been recognized that the quality of products for human consumption can be significantly affected by the presence of pathogenic microorganisms. Such products include drinking water, bathing water, food, food preparation equipment, and, in general, any vector which the microorganisms may use to invade the human body. If present in sufficient numbers, such microorganisms can cause product deterioration or spoilage, disease, and significant economic loss. However, conventional processes for detecting such microorganisms in such products generally require twenty-four to seventy-two hours to complete. This elapsed time is generally too great to provide information about a decrease in product quality in time to take remedial measures.

Total coliform (TC) bacteria are those normally present in the colon or intestine of humans or animals. Fecal coliform (FC) bacteria are those TC bacteria which are generally present in the feces of humans or animals.

The two former groups (TC and FC) are indicators of sanitary quality. A process for their detection is conventionally considered to be "rapid" if it takes less than twenty-four hours to perform.

Recent development in rapid processes for use in water analyses are presented in Part 919, pp. 1031-1033, Standard Methods for the Examination of Water and Wastewater (APHA, 1985). They are generally not rapid enough to detect a hygienically significant concentration of microorganisms within the ordinary work shift of eight hours.

The prior art includes processes for detecting coliform microorganisms and quantifying the concentration thereof by detecting and quantifying the enzymes which the microorganisms give off as they metabolize. The enzyme in turn is detected and quantified by its ability to cleave certain chemicals, known as fluorogenic substrates, into products. At least one of these products, the fluorescent portion, fluoresces; that is, it emits light at one wavelength when irradiated with light of a different wavelength. The fluorogenic substrate, however, does not fluoresce. The presence of fluorescence therefore indicates the presence of bacteria. See, e.g., Findl et al, U.S. Patent No. 4,242,447.

An example of the aforementioned fluorogenic substrates are 4-methylumbelliferone (4-MU) compounds. The 4-methylumbelliferone product fluoresces upon excitation with a light with a wavelength of about 365 nm to emit a light with a wavelength of about 465 nm. MU derivatives have been used in the prior art. Snyder et al., Appl. Environ. Microbiol., 51 (5): 969-977 (1986); Feng et al, Appl. Environ. Microbiol., 43 (6): 1320-1329 (1982); and Koumura et al., U.S. Patent No. 4,591,554.

The Koumura et al patent discloses a method for detecting microorganisms in a sample by means of fluorescence analysis of umbelliferone derivatives. In the method, microorganisms are incubated with a solution containing lactose and a non-fluorescent umbelliferone derivative until a fluorescent umbelliferone derivative is liberated which in turn is measured and related to the number of microorganisms in the solution. The disclosed microbial inspection can be carried out within one to twelve hours (line 44, col. 2) if the solution contains 10^4 microorganisms per ml (lines 44-49 and 62-64, col. 3). Cells may be ruptured to release the fluorescent moiety. For solutions having fewer than 10^4 organisms/ml, the disclosure suggests culturing the solution with a nutrient medium for one to twelve hours to increase the organism concentration above 10^4 cells/ml. Additionally, the solution of microbial cells could be increased to the 10^4 cell/ml minimum by concentrating the solution.

The Koumura et al. reference cites a wide range of temperatures (10° to 60°C) and times (10 minutes to 6 hours) at which to incubate the mixture. However, in none of the examples cited in the Koumura et al. reference was the umbelliferone incubation time less than one hour. In an example (Example 5) representative of those involving coliform bacteria, after one hour of incubation with an umbelliferone derivative, the only sample producing detectable amounts of 4-MU, contained 4×10^4 cells/10 ml, i.e. 4×10^3 cells/ml (Table 6; one hour cultivation period).

The Koumura et al. reference relies on intracellular enzymes but teaches that sensitivity of the method could be increased several times by rupturing (lysing) the microbial cells prior to exposure to umbelliferone (lines 65-68, col. 3; lines 1-5, col. 4). The Koumura et al. reference also discloses a method in which cell numbers are propagated as an inherent aspect of the method, for example, increasing as much as fourfold after one hour of incubation (Table 6).

The Koumura et al. reference does not disclose a method in which coliform bacteria can be fluorometrically quantitatively detected from suspensions having fewer than about 10^4 cells/ml (Col. 3, lines 62-64 and col. 4, lines 10-12). The Koumura et al. reference does not disclose a method effective in less than one hour.

Dorn, in U.S. Patent No. 3,928,139, discloses a method of rapid quantitative detection of microbial pathogens in a sample fluid by concentrating the pathogens in the fluid in a liquid filtering medium placed under centrifugation. The liquid filter medium into which the pathogens pass can be added to nutrient media for culturing.

"Fluorescence" can be measured by measuring the amount of fluorescence after a given incubation time, or by measuring the incubation time needed to obtain a given amount of fluorescence. Processes which do this generally take enough time for the microorganisms to multiply exponentially through several generations. There is, therefore, an exponentially larger amount of fluorescence available to be measured at the end of the incubation period, whether that period is fixed or variable.

"Fluorescence" can also be measured by frequently sampling the amount of fluorescence during the first part of the incubation period, and calculating therefrom the fluorescence velocity; that is, the time rate of change in the amount of fluorescence. Processes which do this generally do not take enough time for the microorganisms to multiply exponentially through several generations. Instead, such processes rely on the tendency of the microorganisms which are initially present to produce the same amount of enzyme during each sample period, thereby causing a linearly increasing amount of fluorescence. See Snyder et al., above.

Such rapid processes have many benefits. First, they are, by definition, more rapid than those which wait several generations. Second, their accuracy is good, for two reasons. One, they measure current activity of the initially present microorganisms, rather than having to make deductions from measurements of the activity of microorganisms several generations later. Two, they generate multiple data points, a situation which lends itself well to linear regression analysis, rather than generating the single datum produced by the slower processes. Third, the linearity of the data points (or lack of it) is an indicator of the accuracy of the measurement.

Such rapid processes have a drawback as well. The microorganisms must be concentrated enough to produce a measurable amount of fluorescence velocity in less than a generation. When further concentration is not practical, means must be found which enhance the per-organism fluorescence velocity.

It is therefore an object of the present invention to provide a process which enhances the per-organism fluorescence velocity of an enzymatic measurement of a dilute sample of coliform microorganisms in a product for human consumption.

It is a further object of the present invention to enhance such velocity while using 4-methylumbelliferone as the fluorescent product.

The invention comprises a process for assaying living coliform bacteria in a liquid or liquified sample for human consumption having a bacterial concentration as low as one bacterium per 100 milliliters, and in a time of at least six hours, the process comprising: concentrating the microorganisms on a filter, contacting the microorganisms with an actuating medium, incubating and irradiating the mixture, characterized in

(a) concentrating the bacteria upon a filter having pores sufficiently small to retain the bacteria;

(b) placing the filter and bacteria held thereby against a culture medium in a container, the culture medium comprising:

(1) a nutrient for supporting metabolism and reproduction of the bacteria;

(2) a production agent for inducing the production of an enzyme in said bacteria when the bacteria are metabolizing;

(3) a fluorogenic substrate for reacting with the enzyme to release 4-methylumbelliferone from the fluorogenic substrate; and

(4) sodium lauryl sulphate effective in enhancing fluorescence;

(c) incubating the culture medium, filter and bacteria under conditions which allow, during the period of incubation:

(1) metabolism and reproduction of the bacteria,

(2) production of the enzyme,

(3) reaction of the enzyme with the fluorogenic substrate, and

(4) release of sufficient of 4-methylumbelliferone from the fluorogenic substrate from each single bacterium and its descendants to form a visible microcolony under fluorescent conditions;

(d) irradiating the microcolonies with light of a wavelength sufficiently close to that of an excitation wavelength characteristic of the fluorescent portion, and sufficiently intense, as to cause the microcolonies to fluoresce; and

(e) counting the number of fluorescent microcolonies.

In a preferred embodiment, the sample is liquid or has been liquefied, and the actuating medium is liquid. The preferred embodiment further comprises, before the contacting step, concentrating the sample by filtering it through a filter fine enough to retain the microorganisms, the subsequent contacting step being accomplished by placing the filter in the actuating medium.

In a preferred embodiment, the production agent comprises lactose, the permeability agent comprises sodium lauryl sulfate, and the fluores-

cent portion 4-methylumbelliferone, is caused to fluoresce with emitted light of a wavelength of about 465 nm by being excited with light of a wavelength of about 365 nm.

When it is desired to measure coliform bacteria in a concentration of at least 60 coliforms per 100 milliliters in at least 15 minutes, the enzyme comprises β-D-galactosidase, and the fluorescent substrate comprises 4-methylumbelliferone-β-D-galactoside. For total coliforms the environment comprises a temperature of about 35 °C. For fecal coliforms, the environment comprises a temperature of about 41.5 °C.

In accordance with a second embodiment of the present invention, a process for assaying living coliform microorganisms in a liquid or liquefied sample of product for human consumption in a concentration of at least 1 microorganism per 100 milliliters, and in a time of at least six hours, comprises five steps.

The first step is filtering the sample through a filter fine enough to retain the microorganisms. The second step is placing the filter and microorganisms against a solid or at least semi-solid substance. The substance comprises the same constituents as are comprised in the actuating medium of the first embodiment of the present invention.

The third step is incubating the substance, filter, and microorganisms in an environment which allows, during the period of incubation, metabolism and reproduction of the microorganisms, production of the enzyme, contacting of the enzyme (whether inside or outside the microorganisms) with the fluorogenic substrate, and release of sufficient of the fluorescent portion of the fluorogenic substrate from each single microorganism and its descendants to form a visible microcolony under fluorescent conditions.

The fourth step is irradiating the microcolonies with light of a wavelength sufficiently close to that of an excitation wavelength characteristic of the fluorescent portion, and sufficiently intense, as to cause the microcolonies to fluoresce. Finally, one simply counts the number of fluorescent microcolonies.

In a preferred embodiment an alkaline solution is added to the microcolonies at the end of the incubation period, thereby enhancing fluorescence and facilitating counting; and the substance against which the filter and microorganisms is placed comprises agar. The remainder of the preferred embodiment of this second embodiment is the same as the remainder of the preferred embodiment of the first embodiment, and detects the same microorganisms.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other important features of the present invention will be better understood from the following detailed description, made with reference to the following drawings, in which:

FIG. 1 is a graph showing fluorescence production by coliform bacteria in drinking water contaminated with raw sewage (solid circles), with the addition of lactose (open circles), and with the addition of sodium lauryl sulfate (triangles);

FIG. 2 is a graph correlating fluorescence velocity with total coliform concentration, with error bars indicating the range obtained when replicate samples (open circles) are incubated from one original sample;

FIG. 3 is a graph similar to FIG. 2, except that it correlates fluorescence velocity with fecal coliform concentration rather than with total coliform concentration;

FIG. 4 is a photograph of the agar of the second embodiment, showing individually fluorescing microcolonies;

FIG. 5 is a graph correlating measurements obtained from the agar process with comparable measurements obtained from the conventional M 7h FC process.

The present invention makes use of the enzyme activity in the coliform group of bacteria to detect their presence and to quantify them. Enzymes unique to a group of microorganisms can be further used to identify the group to the exclusion of others present in the sample.

Specifically, the present invention uses enzyme activity as determined by the detection of MU-derived enzyme products to detect and quantify the coliform group of bacteria.

As noted above, the coliform group includes the total coliforms (TC) and the fecal coliforms (FC). These are considered indicators of the sanitary quality of food, water, or process equipment; that is, their presence is generally thought to indicate contamination by fecal material. The coliform group constituents possess the enzyme β-D-galactosidase. The present invention detects this enzyme by the use of 4-MU-β-D-galactoside in the presence of known selective ingredients to exclude the activity and presence of any other β-D-galactosidase positive non-coliforms. TC and FC are differentiated on the basis of temperature: TC are detected at 35 °C; and FC are detected at 41.5 °C.

The general procedure for the detection of TC or FC activity in the present invention is as follows:

(a) the sample is concentrated by passing it through a membrane filter (0.2 μm to 0.80 μm pore size);

(b) the microorganisms which are retained with the filter are aseptically placed in contact with a

sterile medium containing the appropriate 4-MU-substrate; and

(c) the resulting fluorescence is measured and utilized as the rate of production of fluorescent product in the liquid medium associated with the sample determined at regular intervals over about fifteen minutes using a fluorescence detecting meter.

TC are detected in the manner described above by incubation of the sample plus 4-MU-β-D-galactoside at 35 °C, whereas FC are detected by like incubation at 41.5 °.

The invention is illustrated by the following examples.

EXAMPLE 1

Total coliform (TC) bacteria were analyzed in the following manner. Natural populations of coliforms were obtained from a river (Nidelven, Trondheim. Norway) receiving sewage effluent and from fresh raw domestic sewage. Samples were collected daily for experiments and diluted in tap water in varying concentrations to model drinking water with fecal coliform (FC) and nonspecific heterotrophic plate count (HPC) bacterial contamination. Concentration of coliforms ranged from 1/100 ml to 10000/100 ml as indicated in FIG. 2.

500 ml of a water sample were filtered through a 0.45 µm pore size 47 mm diameter membrane filter (Millipore) and aseptically placed in a 250 ml flask containing 18 ml of sterile buffered actuating medium. (The 500 ml sample size was chosen as the maximum volume of surface water of turbidity < 5 NTU that could be filtered in a short period of time). The medium consisted of phosphate buffered saline (PBS; pH 7.3), 0.02% sodium lauryl sulfate (Sigma Chemical Co., St. Louis, Missouri), 0.56% nutrient broth (Difco Laboratories, Detroit, Michigan), and 0.35% lactose. One of the fluorogenic substrates was added to each flask and to a sterile control flask containing the sterile medium, then placed in a shaking water bath. 4-methylumbelliferone-β-D-galactoside (4-MU-β-D-galactoside) was added at a concentration equal to 0.05 mg/ml.

The samples were incubated at 35 °C for determining TC activity, and at 41.5 °C for determining FC activity. Flasks were removed from the bath and placed in a fluorimeter (Turner Model 111) every five minutes for thirty minutes to measure the initial fluorescence velocity. The excitation and emission wavelengths for methylumbelliferone are about 365 and 465 nm using light filters 7-60 and 2A + 47B respectively. Fluorescence velocity was determined by least squares linear regression.

The rapid fluorescence processes were compared with the conventional enumeration techniques described below. Heterotrophic plate count (HPC) bacteria were recovered on SPC (Difco) or R2A agar and counted after three days incubation at 20 °C. Total coliform (TC) bacteria were assayed with the membrane filtration technique using m-ENDO agar (Difco) and incubated for twenty-four hours at 35 °C. The seven hour and standard twenty-four hour membrane filtration processes (APHA, 1985) were used to recover fecal coliform (FC) at 41.5 °C and TC at 35 °C respectively. Peptone (0.1%) was used for all serial dilutions. All assays were done according to Standard methods - (APHA, 1985).

Production of methylumbelliferone from 4-MU-β-D-galactoside by coliform bacteria in drinking water contaminated with raw sewage (solid circles) is shown in FIG. 1. Addition of lactose enhanced induction of β-D-galactosidase (open circles).

Addition of sodium lauryl sulfate, a common selective ingredient for coliforms, further enhanced activity (triangles). Enzyme assay temperature was 35 °C.

The addition of lactose and sodium lauryl sulfate to the medium enhanced galactosidase activity. The specificity of MU-galactoside for members of the coliform group was tested by pretreating water samples with common inhibitors employed in coliform media (Standard Methods, 1985). Most of the activity was retained after pretreatment suggesting that the galactosidase activity was attributable to the coliform group as defined by the conditions of the assay. Replacement of sodium lauryl sulfate by the selective agents used in mT7h medium, polyethylene ether WU-1 and tergitol 7 did not enhance activity (data not shown). Linearity of the rate of product formation was generally high, r = .99 for most assays. Therefore the duration of the assay in the remaining experiments was decreased from 120 minutes to 30 minutes, although 15 minutes was sufficient.

FIG. 2 shows the correlation between initial rate of hydrolysis of 4-MU-β-galactoside, as measured by initial fluorescence velocity, and the concentration of total coliform bacteria derived from raw sewage and mixed in drinking water. Enzyme assay temperature was 35 °C. Error bars associated with open circles represent the range of fluorescence velocity obtained when replicate samples (5 < n < 8) are incubated from one original sample.

EXAMPLE 2

Fecal coliform (FC) bacteria were determined in the same manner as in Example 1 except that an incubation temperature equal to 41.5 °C was used.

The results are shown in FIG. 3. Initial rate of hydrolysis of 4-MU-β-D-galactoside by fecal coli-

form bacteria derived from raw sewage and mixed in drinking water, as measured by initial fluorescence velocity, is compared with fecal coliform bacterial concentration. Enzyme assay temperature was 41.5 °C. Error bars associated with open circles have the same meaning as in FIG. 2.

EXAMPLE 3

This example demonstrates direct counting of FC. Concentrations of FC < 100/100 ml were analyzed by direct counting. Water samples were filtered and placed on a solid or at least semi-solid substance, namely M 7h FC agar (Standard Methods, 1985) lacking the pH indicators and d-mannitol but containing 4-MU-β-D-galactoside. Fluorescent galactosidase positive microcolonies were visible after six hours incubation at 41.5 °C with a 366 nm long wave ultraviolet light source (UVL-56, Ultraviolet Products, San Gabriel, California). The addition of 0.2 ml of an alkaline solution, namely 0.1 N NaOH, after the incubation period increased the fluorescence and facilitated counting.

FIG. 4 shows an example of the appearance of the microcolonies after six hours of incubation. FIG. 5 compares concentrations as measured by the agar direct counting process with concentrations measured by the conventional M 7h FC process. Pluses represent raw sewage contaminated drinking water stored at 15 °C and sampled after twenty-four to seventy-two hours for coliforms, and solid circles represent river water samples. The dashed line represents the ideal correlation, and is not fit to the data. The average ratio of MU 6h MF to M 7h FC was 1.21 to 1 for thirty-four samples.

Sixty-two fluorescing colonies randomly selected from three experiments showed 100% confirmation as FC by gas production in lauryl tryptose broth incubated at 35 °C for twenty-four hours and in EC broth incubated at 44 °C for twenty-four hours. In all experiments HPC concentrations ranged from 300/ml to 50,000/ml.

Claims

1. A process for assaying living coliform bacteria in a liquid or liquefied sample of product for human consumption having a bacterial concentration as low as one bacterium per 100 milliliters, and in a time of at least six hours, the process comprising: concentrating the microorganisms on a filter, contacting the microorganisms with an actuating medium incubating and irradiating the mixture, characterized in

   (a) concentrating the bacteria upon a filter having pores sufficiently small to retain the bacteria;

   (b) placing the filter and bacteria held thereby against a culture medium in a container, the culture medium comprising:

   (1) a nutrient for supporting metabolism and reproduction of the bacteria,

   (2) a production agent for inducing the production of an enzyme in said bacteria when the bacteria are metabolizing,

   (3) a fluorogenic substrate for reacting with the enzyme to release 4-methylumbelliferone from the fluorogenic substrate, and

   (4) sodium lauryl sulphate effective in enhancing fluorescence;

   (c) incubating the culture medium, filter, and bacteria under conditions which allow, during the period of incubation:

   (1) metabolism and reproduction of the bacteria,

   (2) production of the enzyme,

   (3) reaction of the enzyme with the fluorogenic substrate, and

   (4) release of sufficient of 4-methylumbelliferone from the fluorogenic substrate from each single bacterium and its descendants to form a visible microcolony under fluorescent conditions;

   (d) irradiating the microcolonies with light of a wavelength sufficiently close to that of an excitation wavelength characteristic of the fluorescent portion, and sufficiently intense, as to cause the microcolonies to fluoresce; and

   (e) counting the number of fluorescent microcolonies.

2. A process according to claim 1, wherein the culture medium comprises a nutrient for supporting metabolism and reproduction of the bacteria on the filter.

3. The process according to claim 1 and/or 2, wherein the step of incubating further comprises adding an alkaline solution to the microcolonies at the end of the incubation period, whereby fluorescence is further enhanced for facilitating counting.

4. The process according to one or more of claims 1-3, wherein in the step of placing the filter and the bacteria on the substance, the production agent is lactose, the enzyme is B-D-galactosidase; the fluorescent substrate is 4-methylumbelliferone-B-D-galactoside, the culture medium against which the filter and bacteria is placed is agar and the fluorescent portion is caused to fluoresce with emitted light of a wavelength of about 465 nm by being

excited with light of a wavelength of about 365 nm.

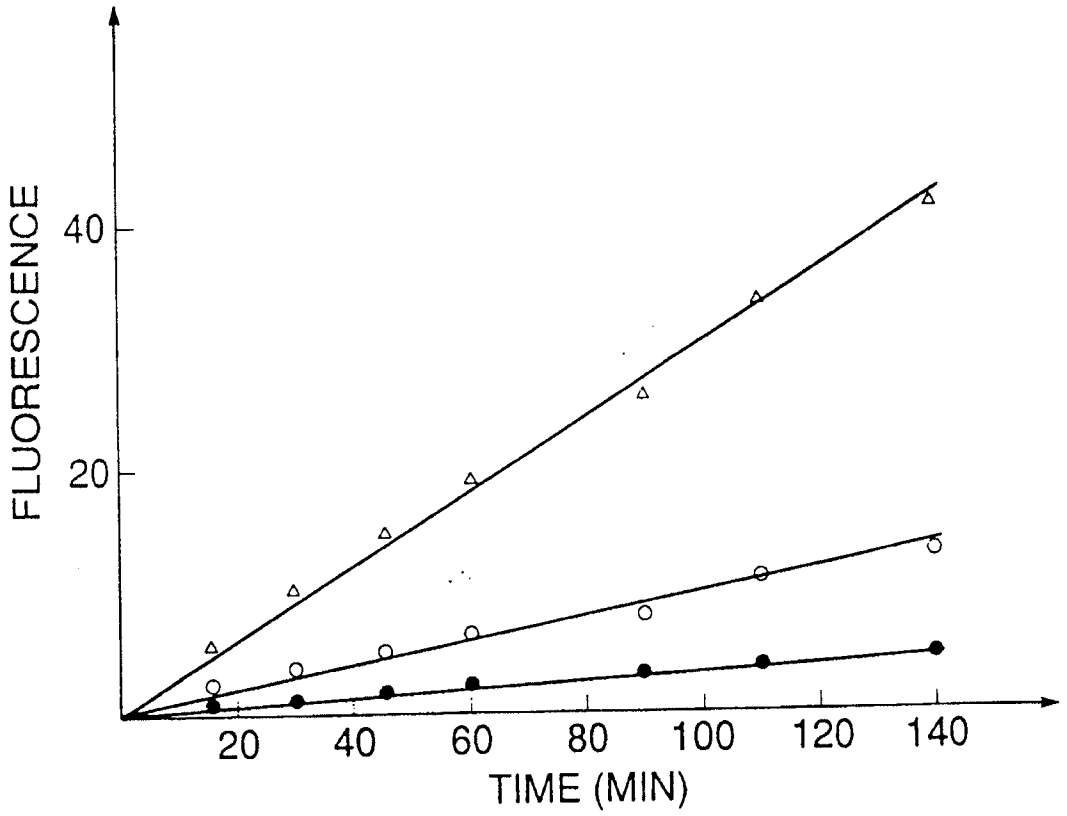
5. The process according to one or more of claims 1-4, wherein the bacteria are total coliforms and the incubation temperature is about 35 °C.

6. The process according to one or more of claims 1-4, wherein the bacteria are fecal coliforms and the incubation temperature is about 41,5 °C.

7. The process according to one or more of claims 1-6, wherein the incubation step is carried out by using a water bath.

8. The method according to one or more of claims 1-7, wherein in the step of concentrating the bacteria upon a filter, the filter has pore sizes in a range of from 0,2 µm to 0,80 µm in diameter.

FIG. 1.



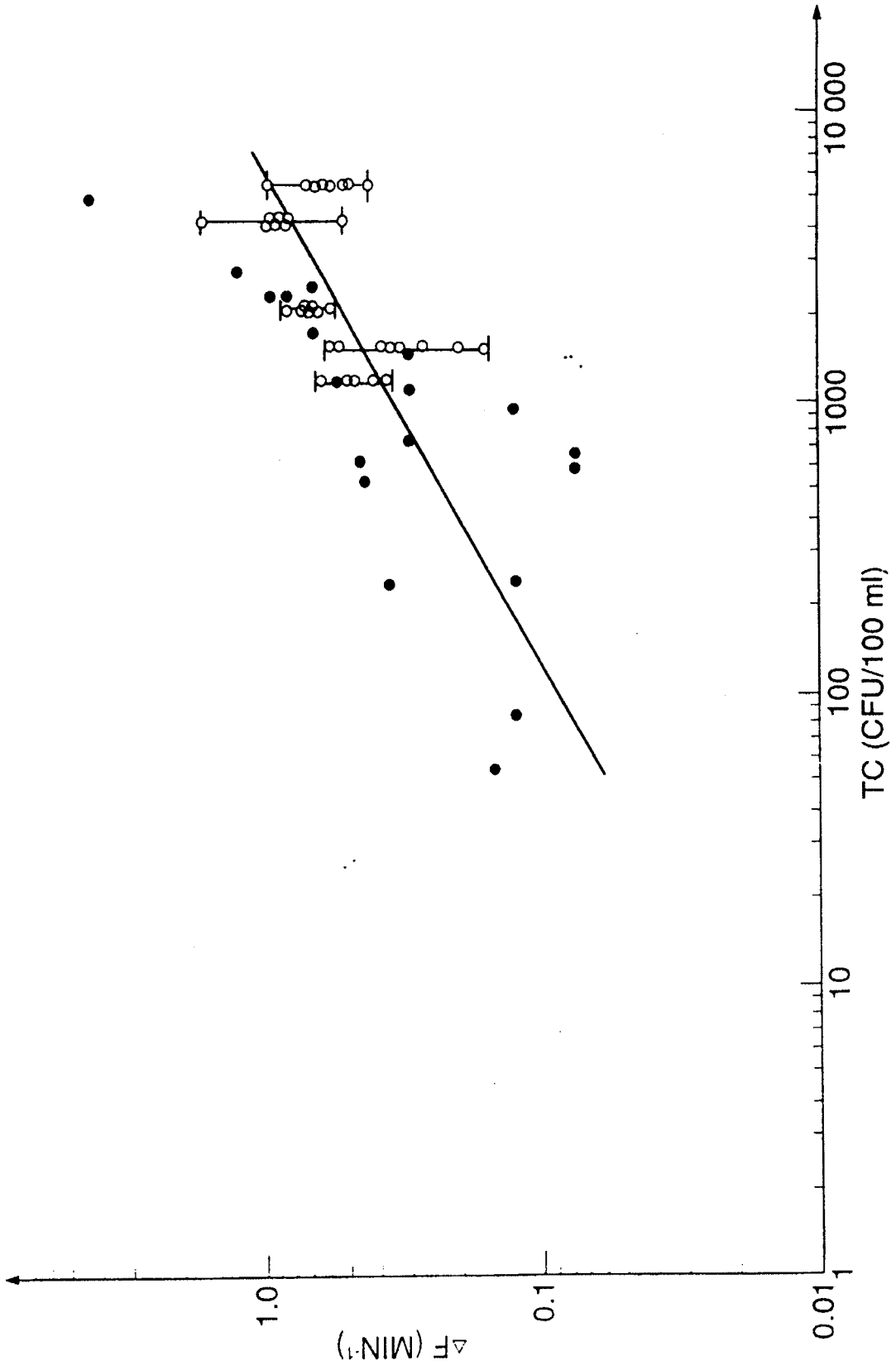


FIG. 2.

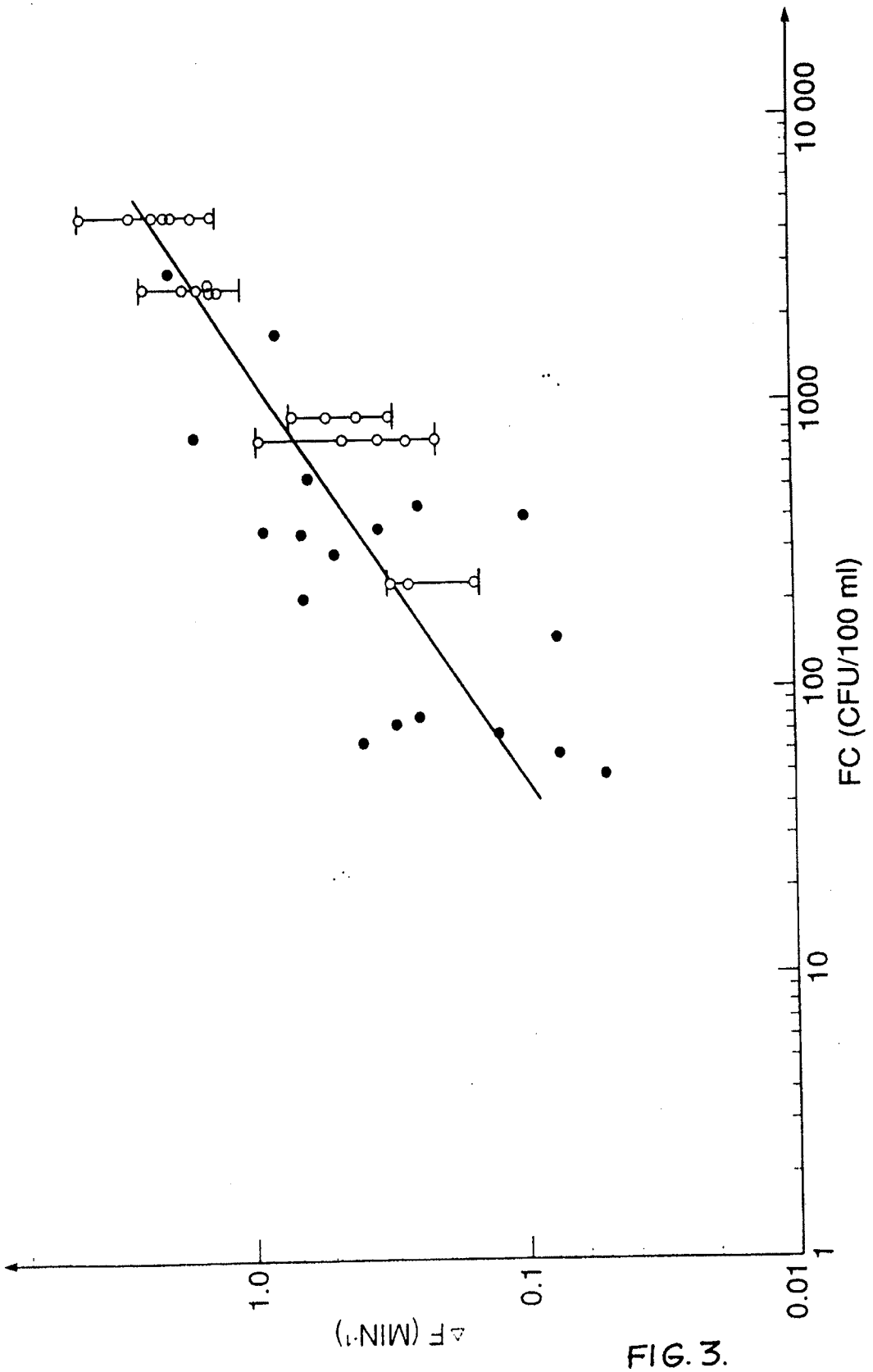
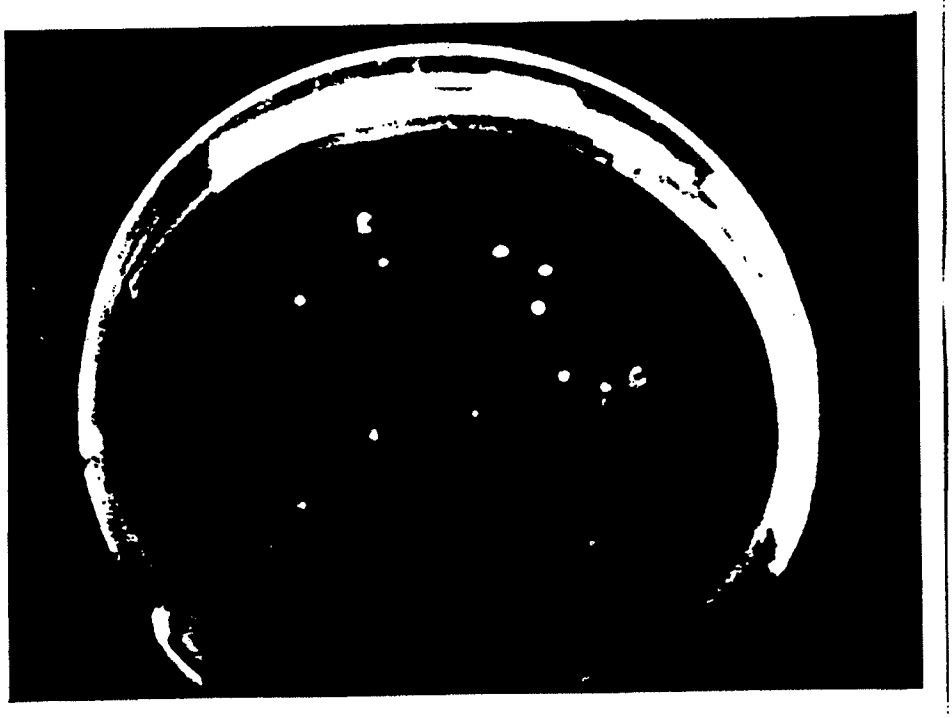


FIG. 3.

FIG. 4.



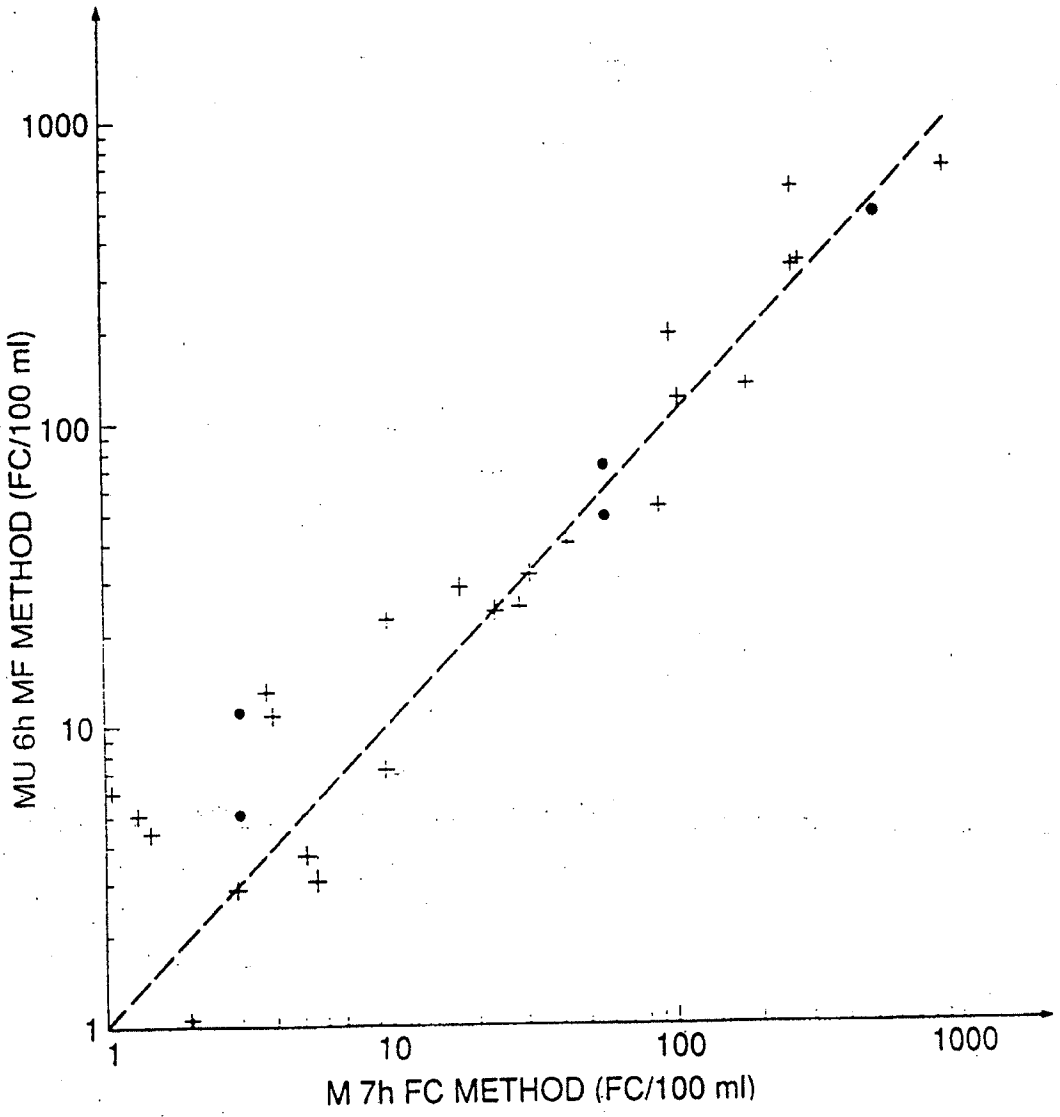

1000
100
10
1
MU 6h MF METHOD (FC/100 ml)
1
10
100
1000
M 7h FC METHOD (FC/100 ml)

FIG. 5